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Institut für Immunologie

Role of Viral Infection in Transplantation Medicine

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Folgende Publikationen beinhalten Teile der Dissertation:

***IL-10* induces T cell exhaustion during transplantation of virus infected hearts**

Gassa A, Jian F, Kalkavan H, Duhan V, Honke N, Shaabani N, Friedrich SK, Dolff S, Wahlers T, Kribben A, Hardt C, Lang PA, Witzke O, Lang KS

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High frequencies of anti-host reactive CD8+ T cells ignore non-hematopoietic antigen after bone marrow transplantation in a murine model

Gassa A, Kalkavan H, Jian F, Duhan V, Khairnar V, Shaabani N, Honke N, Carpinteiro A, Botezatu L, Crivello P, Dolff S, Ferencik S, Häussinger D, Khandanpour C, Fleischhauer K, Witzke O, Wahlers T, Hardt C, Lang PA, Lang KS

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Contents

1 Introduction

The immune system is a powerful means against a broad spectrum of infectious agents in the environment, such as viruses, bacteria, fungi and prions. Successful elimination is guaranteed by two fractions which can interact intensely: On the one hand, the innate immune system provides granulocytes and macrophages that follow physiological barriers such as skin and mucous membrane. On the other hand, delayed but specific immune response is maintained by the adaptive immune system (Dempsey et al., 2003). Immune cells from both sections originate from hematopoietic stem cells in the bone marrow. A central capacity of the immune system is that it can distinguish between self and non-self-antigens (Jiang et al., 2009). In case of transplantation, a fully competent immune system recognizes foreign antigens on transplant tissue and in consequence, leads to rejection of the transplant. To avoid rejection of transplant tissue, suppression of the host immune system has to be maintained.

1.1 The Adaptive Immune System

The adaptive immune system differs from the innate immune system by its specific and delayed immune response. The adaptive or acquired immune system is also capable of memorizing experienced infections which lead to a fast and efficient elimination of invasive pathogens. Cellular components of this very specific immune response are antigen presenting cells (APCs), T cells and B- cells. Lymphocytes develop from hematopoietic stem cells and expose antigen specific receptors which recognize peptides and proteins. During maturation of lymphocytes somatic recombination of antigen specific receptors enables a highly specific B cell and T cell receptor repertoire. Theoretically, 10^{14} different B cell receptors (BCR) and 10^{18} different T cell receptors (TCR) are available (Lehrer, 2004). In general, two types of immune responses can be differentiated: the B cell derived humoral immune response and the T cell mediated cellular immune

response. Here in this study we focused on T cell mediated immune response and their interaction with foreign antigens.

1.1.1 Antigen Recognition by T Cells

T cells develop from common lymphoid progenitors (CLP) in the bone marrow and mature in the thymus. The TCR is a transmembrane protein, composed of two chains, the alpha and beta chains, which are associated with the CD3 complex (Borst et al., 1996; Call et al., 2005). Recognition of foreign antigens is only possible when peptides are exposed on the surface of the body's own cells. Foreign antigens can originate from viruses or intracellular bacteria which are processed intracellularly and then exposed on the surface; similarly for other pathogens or their fragments which are processed after endocytosis. T cells identify infected cells only if foreign peptides are presented on host specific glycoproteins which are defined as major histocompatibility complex (MHC) molecules. Discovery of highly polymorphic genes located in the MHC explained the powerful immune response to transplanted tissue (Murphy, K.P. (2012): Janeway's Immunobiology. 8th Ed. New York).

1.1.2 Major Histocompatibility Complex (MHC)

Characteristic for T cell recognition of antigens is the TCR which interacts with MHC surface molecules. MHC molecules are expressed on lymphoid and non-lymphoid tissue except on erythrocytes. There are two types of MHC: MHC class I and MHC class II. Their function is to present foreign antigens on the cell surface to T cells so that viral and / or bacterial replication in cells can be stopped by cell killing. Peptides derived from the cytosol are transported to the endoplasmic reticulum where they fuse with newly synthesized MHC class I molecules. This complex is exposed on the cell surface of APCs which present foreign antigens to $CD8^+$ T cells. Peptides generated by degradation of proteins in intracellular endosomal vesicles combine with MHC class II molecules. $CD4^+$ T cells then

recognize the complex of peptide and MHC class II molecules on the cell surface of APCs. $CD8^+$ T cells recognize antigen in the context of MHC class I molecules whereas $CD4^+$ T cells recognize antigen in the context of MHC class II, a process which is called MHC-restriction. $CD4^+$ and $CD8^+$ T cells recognize foreign antigen on self-MHC molecules (self MHC-restricted) or self or foreign antigen on foreign-MHC molecules (allo MHC-restricted). Genetically, MHC molecules are encoded on chromosome 6 in humans and on chromosome 17 in mice and are highly polymorphic which makes it difficult for viruses and bacteria to evade the immune response ("Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium," 1999).

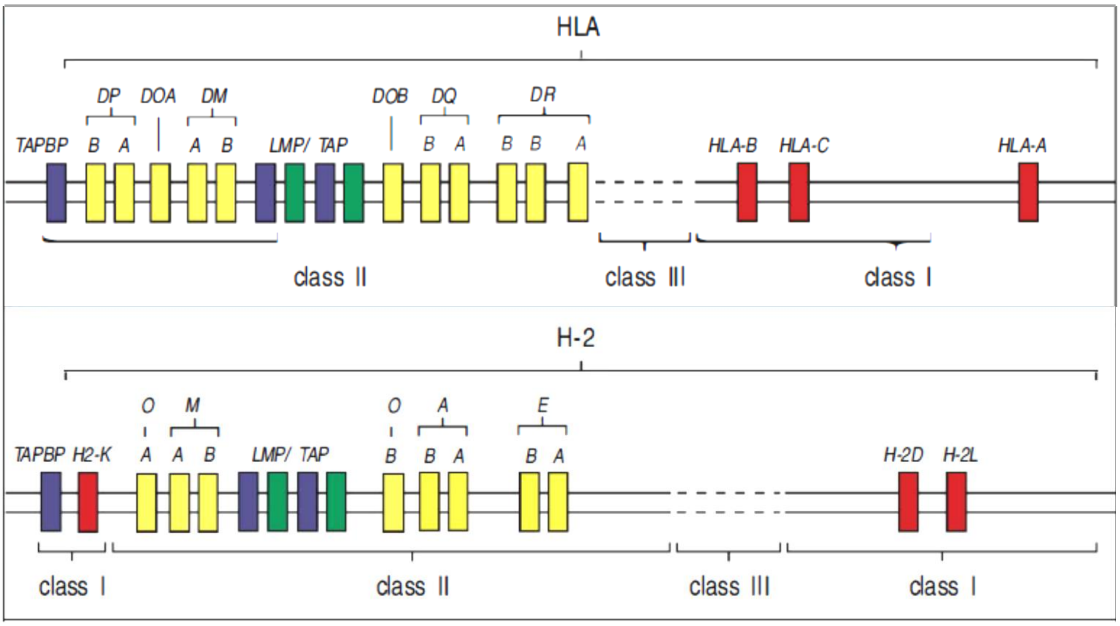

Figure 1.1 Genetic organization of the major histocompatibility complex in human and in mouse. (Murphy, K.P. (2012): Janeway's Immunobiology. 8th Ed. New York. Chapter 6)

1.1.3 Cytokines

Cytokines are key molecules which enable cells to communicate and to interact via cytokine secretion. Cytokines are small proteins that have specific impact on target cells. Cytokines vary in their names depending on their origins. Lymphokines, are cytokines made by lymphocytes, monokines come from monocytes, chemokines are named due to their chemotactic activity and interleukins are cytokines which are made by leucocytes and serve as cell interacting molecules between leucocytes (Moreno Brea et al., 2009). Regulation of suppressive or activating effects in target cells is maintained by pro-inflammatory and anti-inflammatory cytokines. In this study, interferon-γ played a crucial role as pro-inflammatory and interleukin-10 as inhibitory immunosuppressive cytokine.

1.1.3.1 Interferon-γ (IFN-γ)

IFN-γ is one of the different types of interferon besides IFN-α and IFN-β. IFN-γ is also known as type-II interferon, whereas IFN-α and IFN-β belong to type-I interferon. It activates innate immune responses and mediates anti-viral effects. During viral infection, the main sources of IFN-γ production are $CD8^+$ and $CD4^+$ T cells. Signal transduction undergoes the Jak-Stat pathway. IFN-γ mediates activation of macrophages and granulocytes for phagocytosis and cell proliferation (Schroder et al., 2004).

1.1.3.2 Interleukin-10 (IL-10)

IL-10 is an immunoregulatory cytokine that is associated with T cell exhaustion. Its function during viral infection is described to be immunosuppressive and anti-inflammatory by suppressing cytokine production and proliferation of $CD8^+$ and $CD4^+$ T cells, leading to viral persistence. It inhibits NF-κB activation through ill-defined mechanisms. A variety of cells can produce IL-10 such as T cells, B cells and APCs (Blackburn et al., 2007; Moore et al., 2001).

1.2 Lymphocytic Choriomeningitis Virus (LCMV)

LCMV has become an indispensable model in immunology. Due to viral infection experiments in mice, it was possible to start to understand T cell response and MHC-restriction (Zinkernagel et al., 1979, 1997).

1.2.1 Virus Biology

LCMV is an arenavirus, which is a non-cytopathic virus. Regarding the genome, LCMV contains a single stranded RNA. Its physiological reservoir is the house mouse and hamster (Childs et al., 1992). The first arenavirus, LCMV, has been isolated by Charles Armstrong in 1933. During an epidemic study in St. Louis, the virus has been detected. As common house mice provide an LCMV reservoir, human beings can be in contact with the virus. In our days, contamination with LCMV in the population is approximately 5%. Infection with LCMV in humans can be asymptomatic but can also provoke meningitis. In immunosuppressed patients such as transplanted recipients, donor derived LCMV infection in solid organ transplantation (SOT) leads to higher morbidity and mortality. Vertical transmission of LCMV in pregnancy is not well studied but first approach assumes complications and higher morbidity for the fetus.

To get insight into mechanism in viral clearance, different strains of virus exist (Ahmed et al., 1988; Zinkernagel et al., 1986). Concerning the immune response, we distinguish between strains inducing a chronic and an acute infection. Strains as Armstrong and WE enable an acute infection in mice, the virus can be cleared 8 days *post-infection*. Armstrong is a neurotropic LCMV strain whereas WE is a visceral strain. Chronic infections are well described for Clone 13 and docile. Looking closer at the infection with strain WE of LCMV, $CD8^+$ T cell response can be well characterized. Great achievements in immunological understanding of T cell response during viral infection has been done with LCMV. In this study, acute infection in transplantation medicine was the focus. Therefore, LCMV-WE has been used for acute infection in mice.

1.2.2 Immunopathology

LCMV infection is a very good model to study and understand immunopathology. During viral infection, antigen specific $CD8^+$ T cells kill infected cells. During acute infection with LCMV strain WE high dose ($2x10^6$ PFU), T cell mediated tissue damage in liver takes place. Virus can be cleared but at the same time viral infected cells get eliminated. In consequence, tissue damage marker for liver are elevated. Liver enzymes such as alanine transferase (ALT) and bilirubin are elevated. Lactate dehydrogenase (LDH), an unspecific cell damage marker, is massively increased. Chronic infection with LCMV can lead to constant activation of T cells which consequently lead to massive immunopathology and death. In this study, virus titer was determined in organs to look for viral persistence and ALT and LDH were measured in sera (K. S. Lang, Recher, Navarini, et al., 2005; P. A. Lang et al., 2010).

1.2.3 T Cell Exhaustion

T cell exhaustion is defined as a loss of effector function (Wherry, 2011; Yi et al., 2010). Gradual loss can be distinguished in stages which are determined by diminished or vanished production of effector cytokines such as IFN-γ, increased expression of inhibitory receptors and deletion of antigen-specific T cells. Furthermore T cell exhaustion correlates with viral persistence (Wherry et al., 2007).

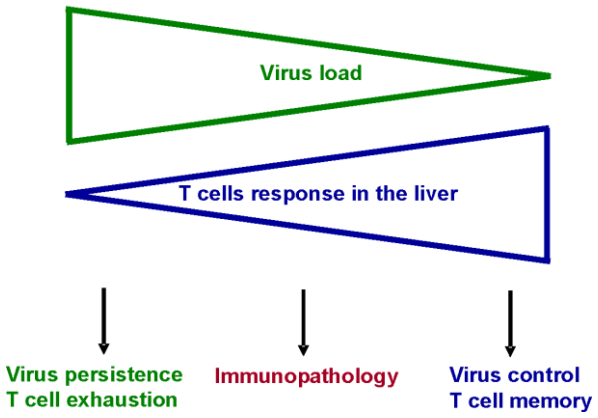

Figure 1.2 T cell exhaustion and immunopathology (P. A. Lang et al., 2010)

1.3 Tolerance in Transplantation Medicine

Immune tolerance is defined as a state of specific immunological unresponsiveness in the immunocompetent host (Dong et al., 1999). Transplantation medicine has improved in the last decades and since the use of new immunosuppressive drugs. Still, long-term survival and chronic rejection remain major challenges in transplantation medicine. Moreover, immunosuppressive drugs bring along many side effects such as toxicity, malignancies and cardiovascular disease (Mihatsch et al., 1998). Therefore, understanding tolerance mechanism in transplantation medicine is crucial.

1.3.1 Bone Marrow Transplantation (BMT)

Hematopoietic stem cell transplantation (HST) is an important therapy for patients with acute myeloid leukemia, myelodysplastic syndrome, refractory relapsed lymphoma and very severe aplastic anemia. HST is the transplantation of multipotent hematopoietic stem cells from bone marrow, peripheral blood, or umbilical cord blood. It can be autologous (patients' own stem cells) or allogenic (donor stem cells). Allogenic stem cell transplantation requires that the transplant carries compatible transplantation antigens which are determined by the variability of three or more loci of the MHC complex. In humans, these MHC class I and class II loci are named human leucocyte antigens (HLA) class I and class II. Once HLA testing is assessed, compatible donors are selected for HST. Compatibility is verified by the two major categories of HLA type (type I and type II). The risk that the transplanted "new" immune system attacks the recipient tissue increases with every HLA-mismatch and causes severe illness. This phenomenon is called graft versus host disease (GvHD). The risk of GvHD increases with mismatches of HLA Type-I genes. Still, fully HLA-matched donor and recipients as in the case of syngeneic HST (siblings) can lead to GvHD.

1.3.2 Solid Organ Transplantation (SOT)

Due to the discovery of HLA in 1967 (Turner, 2004), transplantation medicine progressed enormously. Solid organ transplantation means transplantation of organs and tissues from deceased or living donors. Organs that can be transplanted are the heart, kidney, liver, pancreas, intestine and thymus. Considering tissue transplantation, skin, heart valves, cornea, bones and tendons can be transplanted. Autografts are autologous transplantation within the same person e.g. skin-transplantation. In case of allograft transplantation, certain conditions have to be fulfilled. First of all, as HLA mismatch correlates with allograft rejection, HLA matching between donor and recipient has to be determined. This is assessed by serologic or DNA typing methods.

Furthermore, detection of alloreactive T cells opposed to recipient is measured in mixed lymphocyte reaction (MLR). Histoincompatibility can be verified *in vitro*. If HLA match is determined by DNA typing but MLR reveals histoincompatibility, the donor is not compatible for the recipient. As results usually take a long time to be ready and organ availability is short, HLA match is subordinated to organ demand. Immunosuppression then supports allograft survival by suppressing the immune response nonspecifically. Similar to alloreactive T cells, alloreactive antibodies can be identified in donor-recipient serologic cross matching. This analysis is particularly important for vascularized organs such as kidney and heart. Presence of such antibodies induce hyperacute rejection (Kissmeyer-Nielsen et al., 1966).

1.3.2.1 Heart Transplantation (HTX)

Heart transplantation is the therapy of choice for patients with heart insufficiency. So far, limited amount of available donor organs and the rejection of hearts after transplantation are major problems in heart transplantation. The first heart transplantation was performed in South Africa in 1967. Over the last two decades, selection methods for donor and recipients have increasingly improved. In addition, introduction of immunosuppressive drugs such as cyclosporine

opened up new opportunities in transplantation medicine. Overall, after transplantation, survival in the first year is more than 90% and in the second year approximately 70%. The field of heart transplantation is constantly progressing. Advances in organ preservation, immune monitoring and immunosuppressive regimens are likely to lead to a better outcome and to advances in the quality and the length of life of heart transplant recipients (Hoffman, 2005; Hunt et al., 2008).

1.4 Challenges in Transplantation Medicine

In the last 20 years, transplantation medicine has strongly improved thanks to immunosuppressive drugs, better surgical techniques and controlling infectious disease. Still, lethal problems exist in transplantation medicine and tolerance mechanisms are not understood yet.

1.4.1 Graft versus Host Disease (GvHD)

Still limitation to successful bone marrow transplantation (BMT) comes from severe GvHD, which occurs in up to 40% - 60% of cases (Jacobsohn et al., 2007; Jagasia et al., 2012) and leads to 15% of deaths after HST. GvHD is clinically grouped in an acute and chronic GvHD defined by the time of GvHD onset after HST with a cutoff of 100 days. Mechanistically chronic GvHD (cGvHD) usually involves several factors including B cell activation, production of autoantibodies and absence of T-regulatory cells (Treg) (Schroeder et al., 2011).

Acute GvHD (aGvHD) is characterized by the reaction of $CD8^+$ T cells which are activated by antigens presented on host MHC class I molecules (Ferrara et al., 1991). Therefore, T cell depletion of the transplant prior BM transfer is performed in cases of HLA mismatch or unrelated donors and significantly reduces aGvHD (Ho et al., 2001; Kroger et al., 2002; Remberger et al., 2001). Due to the high number of registered bone marrow donors most of BMT are done in complete MHC class I match. Nevertheless, around 40% of patients develop aGvHD despite complete HLA-matching (Ferrara et al., 2009) and T cell depletion (Champlin et al.,

2000). Preparative regimens are critical in terms of treatment of the primary disease and as prevention of graft rejection. Common conditioning regimens are either myeloablative or non-myeloablative defined by the pancytopenia caused. During the last 20 years, an alternative preparative regimen has been developed. This therapy is called reduced intensity conditioning (RIC) and causes less tissue damage and is associated with less GvHD (Mielcarek et al., 2003). RIC as an intermediate category of regimens is commonly used for elderly people to improve the non-relapse mortality by reducing toxicity of treatment by either whole body irradiation or chemotherapy (Abdul Wahid et al., 2014; Mohty et al., 2010).

1.4.2 Graft Rejection

Allograft rejection is a major complication in solid organ transplantation. It is defined as a destructive immune response against foreign tissue or organ which can be mediated via cellular or humoral immune response. Three categories of allograft rejection can be distinguished according to their mechanism and time of occurrence conferring to guidelines of the international society of heart and lung transplantation (Task Force 2: Immunosuppression and Rejection). Hyperacute allograft rejection happens within minutes or hours after transplantation. It is mediated via preformed antibodies hostile to HLA type I molecules expressed on endothelium. It leads to immediate loss of an allograft.

Further, acute cellular rejection occurs within 6 months in case of HTX. As the name itself explains, mainly T cells are responsible for infiltration into the allograft. APCs of immunocompetent host migrate from foreign donor organ to lymphoid tissue and prime T cells. This in consequence leads to direct allorecognition. Recipients' APCs are also capable to present peptide of allograft to T cells by indirect allorecognition (Davis et al., 2004).

Chronic allograft rejection limits long-term survival of transplant recipients. Beyond the 2nd year after HTX, cardiac graft recipients suffer from cardiac allograft vasculopathy (CAV) in 30% of cases. Chronic rejection is multifactorial. Viral infection or reactivation such as cytomegalovirus (CMV) can cause chronic rejection. Graft rejection can be well determined by biopsy. Heart specific tissue

damage markers such as creatinine kinase-MB (CK-MB) and troponine I (Top I) can give a hint (Dengler et al., 1998). Treatment is very often too late and has to be optimized.

1.4.3 Role of Viral Infection

The main reason for graft rejection is viral infection during heart transplantation. 40% of the heart rejections in humans are associated with infections (Jung et al., 2011; Yusen et al., 2014). Those infections are mainly caused by herpes viruses. Considering viral infection in transplantation, we distinguish between reactivation of latent infections in donor organs or recipients and unexpected transmission of viral infections through organ transplantation which is defined as donor-derived viral infection. Even though, transmission of acute or latent infection to organ transplant recipients occurs only in about 0.2% of transplantation. Once it occurs, it leads to significantly increased morbidity and mortality in the recipients (Grossi et al., 2009). In transplantation medicine, expected viral infections detected by serologic analysis are known for CMV, hepatitis B virus (HBV) and Epstein-Barr-virus (Razonable, 2011). Special prophylaxis and infection management is preserved in this setting. Of greater concern are the unexpected viral infections transmitted via donor organs. Several reports of infectious diseases through SOT are published and estimated number of unreported cases has to be considered. Donor-derived viral infections include a growing number of pathogens such as HBV, herpes viruses, human T- cell lymphotrophic viruses (HTLV) 1 and 2, West Nile virus, Rabies, LCMV, polyomavirus, parvovirus B19 and many other viruses. In most of such transmissions the mortality is high (Ison et al., 2011; Srinivasan et al., 2005; Winston et al., 2014). Detection of infectious disease in donor organs is limited by technical means and by the short time period between death of donor and the use of transplant. So, routinely evaluation of donors for infectious disease is determined by antibody detection. Nevertheless, a time window of false negative detection for infectious disease exists. For example, for hepatitis C virus (HCV) the window from infection to seroconversion is 30-70 days. Improvements have been

done by nucleic acid testing (NAT), so that the window could be reduced. But still, some infections remain undetected (Fishman, 2007; Fishman et al., 2009).

1.5 Aims

Transplantation medicine is one of the most challenging fields in medicine. Mechanisms establishing tolerance towards graft and host respectively in an immunocompetent environment has not been understood yet. Factors influencing tolerance can be on the one hand minor antigen mismatches despite full HLA-matched donor and recipient. Another problem is viral infection during BMT and SOT which emerged being life-threatening complications. In this study reported here, LCMV was a very good model to analyze differences of immune responses towards minor antigen and virus replication during murine bone marrow and heterotopic heart transplantation. The aim of this study was to investigate the interaction between LCMV specific $CD8^+$ T cells and presentation of LCMV peptide or virus. We hypothesized that only presence of replicating virus can break immunological tolerance.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Chemicals were obtained from AppliChem (Darmstadt), Merck (Darmstadt), Roth (Karlsruhe) or Sigma-Aldrich (Munich).

Carboxyfluorescein Succinimidyl Ester (CFSE)	Invitrogen, Darmstadt
Fluorescence Activating Cell Sorter (FACS)	BD Bioscience, Heidelberg
Lysing Solution	BD Bioscience, Heidelberg
Fetal Calf Serum (FCS)	Biochrom AG, Berlin
Isofluran	Delta Select GmbH, Pfullingen
Lipopolysaccharides of *Escherichia coli* (LPS)	Sigma, Munich
TriFast®TRIzol	PeqLab, Erlangen
Mouse IL-10 ELISA Ready-SET-Go!®	eBioscience
QuantiTect Reverse Transcription Kit	QIAGEN, Hilden
SYBR®Green	(AppliedBiosystems, Darmstadt)

2.1.2 Media and Solution

Dulbecco's Modified Eagle's Medium (DMEM)	PAN Biotech GmBH, Aldenbach
DPBS w/o Mg^{2+}, Ca^{2+}	PAN Biotech GmBH, Aldenbach
Phosphate Buffered Saline (PBS)	DPBS, ddH_2O
FACS-Buffer	PBS 2% (v/v) FCS 0.1% (v/v) NaN3 2 mM EDTA

Erythrocyte-Lysis-Buffer	0.5 M NH4Cl2 10mM KHCO3 0.1mM EDTA pH 7.42
EDTA-Solution	0.5 M EDTA pH 8.0
Iscove's Modified Dulbecco's Medium (IMDM)	175 ml dH_2O, 50 ml 9xIMDM, 2-4 ml 2M NaHO, sterile filtration, 5% (v/v) FCS, 2% (w/v) PSG
Overlay (Plaque Assay)	50% (v/v) IMDM Solution 50% (v/v) 2% Methylcellu-lose

2.1.3 Primer

qPCR-WEGPforw (LCMV-GP)	CGA GCA TCA AAG CTG TGT ACA AT
qPCR-WEGPrev (LCMV-GP)	AAA AGG AAG CTG ACC AGT GCT AA
GAPDH	Mm_Gapdh_3_SG QuantiTect Primer Assay (200), Quiagen GmbH, Hilden

2.1.4 Equipment

ELISA Reader	AnthosLabtecInstruments GmbH, Wals-Siezenheim
FACSCalibur	BD Bioscience, Heidelberg
Fluoreszenz-Mikroskop HS BZ-9000	Keyence GmbH, Neu-Isenburg
Kryostat CM 3050S	Leica, Wetzler
NanoDrop ND-1000	Peqlab Biotechnologie GmbH, Erlangen
TissueLyserII	QIAGEN, Hilden
Micro 220R-Centrifuge	Andreas Hettich GmbH, Tuttlingen

2.1.5 Software

FacsDiva v&6.2.1	BD Bioscience
FlowJo 7.6.1	TreeStar Inc., Ashland (OR, USA)
GraphPad Prism5	GraphPad Software, La Jolla (CA, USA)
MicrosoftOffice 2007	MicrosoftCorporation, Redmont (WA, USA)

2.1.6 Antibody

In this study, antibodies were directly coupled with fluorescence. Antibodies were obtained from BD Pharmigen (Heidelberg) and eBioscience (Frankfurt). Flourochromes used were FITC, PE, PerCP, APC, PeCy7, AmCyan and Pacific Blue.

2.1.8 Mice

All mice used in these studies were maintained on the C57BL/6 (B6) genetic background. All animals were housed in single ventilated cages under standard conditions. Animal experiments were authorized by the Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein Westfalen (Recklinghausen, Germany) and performed in accordance with the German law for animal protection. During survival experiments mice which showed a severe phenotype with weight loss (>20% of starting weight), reduced movement, and general sickness have been killed at indicated time points assuming time of death. Besides the C57BL/6 (B6) mice which were used as wild type (WT) mice different mouse strains have been used in these experiments.

DEE mice

DEE mice are transgenic mice that express ubiquitously the glycoprotein of LCMV under the H2k promoter (Hunziker et al., 2003). On the one hand, these mice

served as recipients for the generation of bone marrow chimera mice and on the other hand, the *DEE* hearts were used as donors for the heterotopic heart transplantation.

IL-10$^{-/-}$ mice

IL-10 deficient mice lack secretion of the immune modulatory cytokine IL-10 (Kuhn et al., 1993). As recipient they received a heterotopic transplanted heart.

P14 mice

P14 T cells recognize the LCMV-GP on MHC class I. The P14 mice express an LCMV-GP33-41 specific TCR on CD45.1 positive CD8^{+} T cells (Pircher et al., 1989) and were used for adoptive transfer experiments and as bone marrow donors.

Sv/129 mice

Sv/129 mice have a different background from C57BL/6 mice. They differ in gene loci and are used as two mice strains with different backgrounds for e.g. stroke research (Fujii et al., 1997; Gerlai, 1996).

Tcrb$^{-/-}$ mice

Tcrb$^{-/-}$ mice are genetically engineered immunodeficient mice which lack the T cell receptor beta chain (Funabashi et al., 2001). After intravenous injection of LCMV-WE, the virus persisted in the mice. Their hearts were used as LCMV carrier donor hearts for heterotopic heart transplantation.

2.1.9 Lymphocytic Choriomeningitis Virus (LCMV)

LCMV strain WE originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) was propagated in L929 cells. Mice were infected intravenously in the tail vein. Applied concentration was either 200 plaque-forming units (PFU) or 2x10^{6} PFU. Viral titers were measured in Plaque Assay using mouse fibrosarcoma cell line derived from C57BL/6 mice (MC57 cells).

2.1.10 Statistical analysis

If not differently stated data are expressed as means and S.E.M. Student's t-test was used to detect statistically significant differences between groups. Significant differences between several groups were detected by two-way analysis of variance (ANOVA). The level of statistical significance was set at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.2 Methods

2.2.1 Bone Marrow Chimera

Creating bone marrow chimera mice is a very useful method to get further insights into immunological processes such as influence of donor derived hematopoietic stem cells on immune responses. Especially, in the field of transplantation medicine, bone marrow chimera experiments play a crucial role for tolerance model (Umemura et al., 2001). This model makes it possible to distinguish between effects on the immune system in recipient induced by cells coming from the donor derived hematopoietic or non-hematopoietic system.

2.2.1.1 Preparation of Donor BM

For the preparation of donor bone marrow, one mouse had to be killed to gain bone marrow for transplantation of six mice. Donor and recipients were sex-matched. After disinfecting the dead mouse, the abdominal skin was cut open to reach the legs till the hip. Femur and tibia got totally removed without breaking the bones. Forceps and scissors were necessary to fulfill the procedure. When the bones were ready to be flushed, they had to be cleaned in 70% ethanol for three minutes and washed with PBS. With help of a 21-gauge needle and medium, the bone marrow got flushed after cutting open both sides of each bone. The bone marrow (which looked like red, long, thin tissue) had to be collected in a 50 ml

tube and vortexed into a single-cell suspension to passage through a strainer. Re-suspended in medium, the bone marrow was ready for injection. Around 50 x 10^6 bone marrow cells had to be injected intravenously in the tail vein of each mouse (Holl, 2013).

2.2.1.2 Recipient Preparation

Six to eight weeks old recipients underwent a full body irradiation by X-ray with 9.5 Gy. 24 hours after irradiation, mice received sex-matched bone marrow diluted in medium by tail-vein injection. 30 days after bone marrow transplantation reconstitution of bone marrow took place in the host.

2.2.2 Heterotopic Heart Transplantation (HTX)

Heart allografts were vascularly anastomosed in an intraabdominal location using the technique described previously (Corry et al., 1973; Wu et al., 2006). Graft ischemic time was typically 20–25 min and total operative time was 45–50 min with a success rate (beating hearts) of more than 90%. Rejection of heart grafts was not associated with death of recipients. The graft function was evaluated by palpation of the abdominal wall daily after operation. The function of the donor heart was assessed using a subjective score of 0 to 3 (zero for no beating; 0.5 for very weak beating; one for weak beating; two for moderate beating; three for full beating).

2.2.3 Plaque Assay

In this mouse model, virus titers in organs or sera were determined by Plaque Assay (Battegay et al., 1991; K. S. Lang, Recher, Navarini, et al., 2005). After harvesting and smashing organs in 1ml DMEM supplemented with 5% FCS, each cell suspension was transferred in 96-well plates and diluted with DMEM 5%FCS so that the cells were titrated at a ratio of 1:3. After transferring the samples in a

24- well plate, 200 µl of freshly prepared MC57 cells diluted 8 x 10^5 cells/ml, were added. MC57 cells are susceptible for LCMV infection. Plates were ready for incubation and *Overlay* had to be added between 3 and 5 hours after starting incubation at 37°C and 5% CO_2. Incubation lasted for two days. At day three, cells were stained and evaluated for PFU. PFU were calculated according to the size of the smashed organ; dilution and logarithm of each variable is shown in the graph.

2.2.4 Flow Cytometry

Flow cytometry enables counting of fluorescent marked cells by 4 different lasers.

2.2.4.1 Tetramer Staining

Tetramers were provided by the National Institute of Health (NIH) Tetramer Facility. In short, 20 µl of blood was stained with allophycocyanin (APC)-labeled GP33 MHC class I tetramers (GP33/H-$2D^b$) for 15 minutes at 37°C. After incubation, the samples were stained with anti-CD8 peridinin-chlorophyll-protein-complex (PerCP; BD Biosciences, Franklin Lakes, NJ) for 30 minutes at 4°C. Erythrocytes were then lysed using 1ml BD lysing solution (BD Biosciences); cells were washed once and analyzed by flow cytometry. Absolute numbers of GP33-specific $CD8^+$ T cells per/µl blood were calculated by FACS analysis using fluorescent beads (BD Biosciences).

2.2.4.2 Intracellular Cytokine Staining (ICS)

For intracellular IFN-γ staining, splenocytes and lymphocytes from blood were stimulated with GP33 in the presence of Brefeldin A. After 6 hours, cells were stained for CD8 (eBioscience) for 30 minutes at 4°C, fixed with 2% Formaldehyde for 10 minutes and permeabilized with 1% Triton-X solution, and stained for IFN-γ with anti-mouse IFN-γ antibody (eBioscience) and analyzed by flow cytometry.

2.2.4.3 CFSE labeled Splenocyte Transfer

To follow proliferation of $CD8^+$ TCR- specific T cells in blood, T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), a molecule, which made it possible to track cell proliferation due to decrease of tagged fluorescent molecule measured in FITC by flow cytometry. In this study, spleens of P14 mice have been smashed, suspended in PBS and filtered through a strainer. One spleen was suspended in 10 ml PBS. 5mM of CFSE has been added to the cell suspension and an incubation period of 10 minutes at 37°C followed. To eliminate remaining CFSE, single cell suspension has been washed twice with 10% FCS in DMEM. After discarding the supernatant, labeled cells were resuspended in IMDM to inject 200µl (10^6 cells) intravenously in one mouse.

2.2.5 Depletion of $CD8^+$ T Cells

For $CD8^+$ T cell depletion, 500µg/100µl of anti-CD8 antibody clone YTS 169.4 (Bioxcell) was injected intraperitoneally on day -3 and -1 in bone marrow donor mice.

2.2.6 Triggering of Innate Immunity

Innate immune response was triggered by injecting 200µl of lipopolysaccharides (LPS) intraperitoneally (250µg/ml) in bone marrow chimera mice.

2.2.8 ELISA

Enzyme linked immunosorbent assay (ELISA) has been performed by the Mouse IL-10 ELISA Ready-SET-Go!® reagent kit using 20µl of plasma.

2.2.9 RT-PCR

Total RNA was isolated from organs with the TriFast®TRIzol (PeqLab, Erlangen) isolation method. Tissue samples with a weight range between 50-100mg have been homogenized in a TissueLyser (QIAGEN, Hilden). RNA was reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit (QiAGEN, Hilden). Gene expression analysis was quantified by RT-PCR using SYBR®Green (AppliedBiosystems, Darmstadt) according to the protocol of the manufacturer. Expression levels were normalized against GAPDH and compared between study groups.

2.2.10 Analysis of Liver and Heart Enzymes

Serum and plasma were analysed in the central laboratory at the university hospital Essen for alanine transferase (ALT) and lactate dehydrogenase (LDH) and for CK-MB and Trop I. Serum or plasma has been collected from mice at various time points. 30µl serum or plasma has been diluted in 270µl PBS and were ready for measurement at the central laboratory.

3 Results

3.1 Transgenic LCMV-GP is ubiquitously expressed

To analyze immune responses of donor derived T cells in recipients expressing foreign antigen, *DEE* mice were used. *DEE* mice are transgenic mice which express LCMV-GP ubiquitously under the H2k promoter. The expression of LCMV-GP in the thymus, the organ of central tolerance induction was shown by RT-PCR **(Figure 3.1A)**. LCMV-GP is a minor antigen which is presented on MHC class I. To confirm that the transgene LCMV-GP is presented by MHC class I molecules in host *DEE* mice, adoptive transfer of GP33 specific $CD8^+$ T cells (P14 T cells) was performed. P14 cells showed proliferation in *DEE* mice **(Figure 3.1B)** but not in WT mice, when analyzed *ex vivo*.

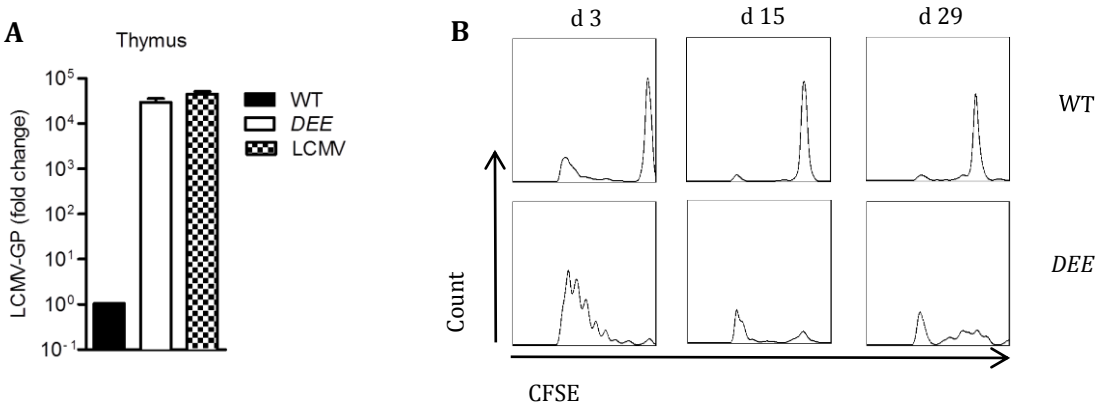

Figure 3.1 LCMV-GP expression in *DEE* mice. A) RT-PCR analysis of LCMV-GP mRNA harvested from thymus of WT (n=2), *DEE* (n=3) and *Tcrb* deficient (LCMV; n=3) mice which were infected with 2 x 10^6 PFU of LCMV-WE representing positive control of LCMV–GP expression due to chronic infection at day 10 *post-infection*. Values show fold change to expression in WT mice. B) Proliferation of P14 T cells. Adoptive transfer of CFSE labeled P14 T cells into naïve WT mice and *DEE* mice were assessed by flow cytometry and shown in histogram blot (n=3).

To emphasize tolerance induction of LCMV-GP specific CD8⁺ T cells in mice carrying the minor antigen LCMV-GP, the F1 generation of P14 mice crossed with *DEE* mice were analyzed for GP33 specific CD8⁺ T cells. Due to negative selection, P14 T cells were deleted centrally **(Figure 3.2)**.

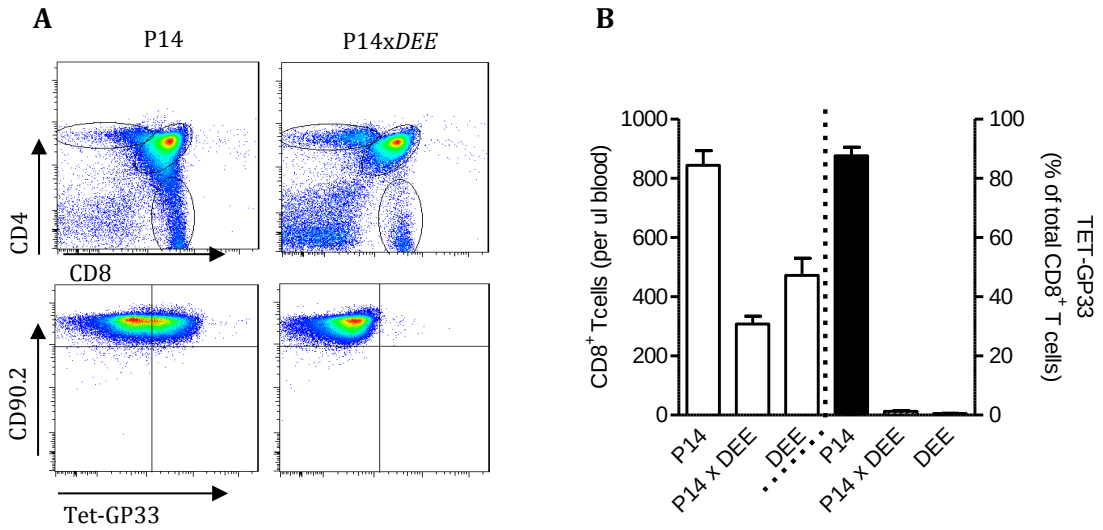

Figure 3.2 Presence of GP33 specific CD8⁺ T cells in thymus and blood. A) Thymus of P14 mice (left panel) and P14x*DEE*mice (right panel) were gated on double positive CD4⁺/CD8⁺ T cells and for double positive CD90.2/Tet-GP33 T cells in the thymus. CD90.2 is expressed by mouse thymocytes and mature T cells. Results are shown as single dot plot. One dot plot is shown as a representative of each group (n=2). B) Number of CD8⁺ T cells in blood was determined in P14, P14x*DEE* and *DEE* mice shown in white columns and percentage of Tet-GP33 positive CD8⁺ T cells in black columns (n=4),

3.2 Host specific CD8⁺ T cells are not negatively selected after BMT

In this murine BMT model, P14 bone marrow was transferred in lethally irradiated *DEE* mice **(Figure 3.3)**. P14 mice revealed LCMV-GP specific CD8⁺ T cells and *DEE* mice expressed LCMV-GP ubiquitously and present the antigen on MHC class I. LCMV-GP was used as a foreign antigen to demonstrate a single minor antigen mismatch.

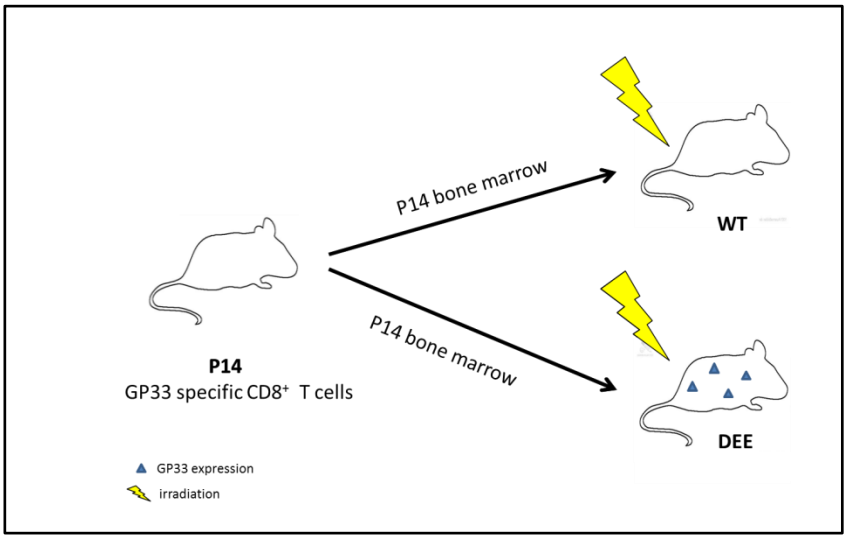

Figure 3.3 Model of BMT. Recipient mice were lethally irradiated (9.5Gy) and received P14 bone marrow intravenously. *DEE* mice expressed GP33 ubiquitously. WT mice recipients were used as control. Mice were at least 6-8 weeks old.

As control, P14 bone marrow was transferred in WT mice. During reconstitution of bone marrow in recipients, blood was analyzed weekly for development of P14 T cells **(Figure 3.4A)**. The graph shows the kinetic of GP33 specific CD8⁺ T cells in *DEE* and WT mice recipients after receiving P14 bone marrow. Even though, *DEE* recipients expressed GP33 everywhere in the tissue and present the antigen on MHC class I, P14 T cells remained ignorant. The mice did not become ill, indeed, measurement of cell damage marker such as LDH and ALT showed a physiological amount in sera **(Figure 3.4B)**.

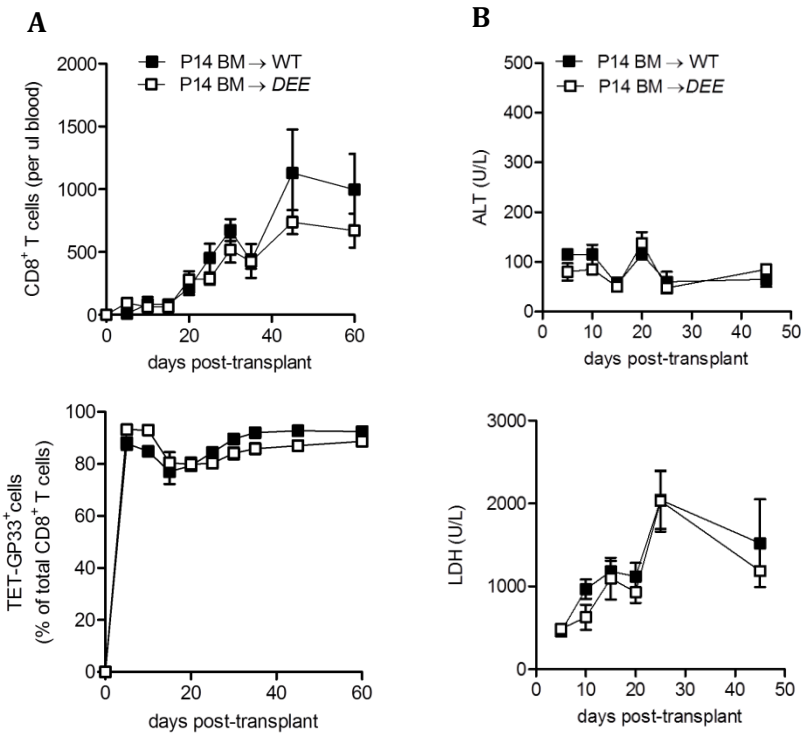

Figure 3.4 Development of P14 T cells after BMT and measurement of ALT and LDH in sera. A) Number of CD8+ T cells in blood was determined weekly in chimera mice by flow cytometry which is shown in the upper panel. According to the total number of CD8+ T cells, Tet-GP33 positive CD8+ T cells are shown in percentage in the panel below. B) ALT and LDH were determined in sera at indicated days post-transplant. Black squares represent mean values of B6 chimera mice at days post-transplant, which received P14 bone marrow *i.v.* (P14 BM-> WT) (n=4). Mean values of T cell number and ALT or LDH of irradiated *DEE* mice receiving P14 bone marrow (P14 BM-> *DEE*) are shown in white squares (n=4).

To characterize ignorant P14 T cells which developed in chimera mice more in detail, spleen has been removed after 40 days as reconstitution of bone marrow took approximately 30 days. Splenocytes were labeled with CFSE and transferred intravenously in different groups of mice. Splenocytes from WT and *DEE* chimera mice were injected *i.v.* in naïve WT mice, naïve *DEE* mice and WT mice infected with LCMV-WE 6 days *post-infection* to see whether P14 T cells behave differently in an environment of viral peptide presentation or active virus replication. P14 T cells which developed either in WT or *DEE* mice were transferred from spleen to WT, *DEE* or LCMV infected mice, proliferated in response to indigenous GP33-peptide (*DEE*) as well as to peptides from replicating virus (LCMV) **(Figure 3.5A)**. *Ex vivo* restimulation of these cells with GP33-peptide to analyze intracellular cytokine staining (ICS) revealed no IFN-γ production in the absence of *in vivo*

antigen exposure (WT), low IFN-γ production after *in vivo* GP33-peptide exposure and significant IFN-γ production after *in vivo* exposure to replicating virus.

In conclusion P14 T cells proliferate in response to GP33-peptide presented in *DEE* mice but they are functionally ignorant. However, if these cells are exposed to an environment with actively replicating virus, these cells can be restimulated and produce IFN-γ.

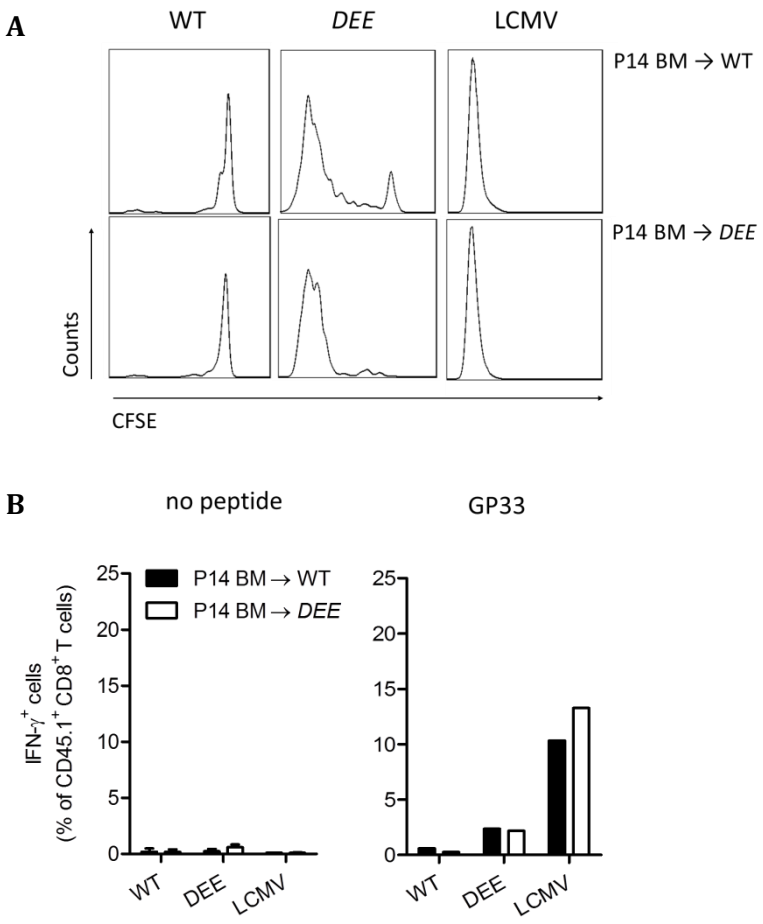

Figure 3.5 P14 T cells generated in chimera mice got activated *ex-vivo*. A) 1 x 10^7 splenocytes of 40-days-old P14 BM→WT mice and P14 BM→*DEE* mice were labeled with CFSE and transferred into naïve WT mice, *DEE* mice and WT mice infected *i.v.* with 2 x 10^4 PFU of LCMV-WE 1 day after splenocyte transfer (n=2-4; pooled from two independent experiments). Proliferation of CD45.1+ CD8+ T cells (P14 T cells) derived from spleen was assessed by CFSE dilution 6 days after transfer. Histograms show cells gated on CD45.1+ CD8+ T cells in correlation to CFSE. One representative set of data is shown. B) Next, these splenocytes were analyzed for IFN-γ production after re-stimulation with the LCMV peptide GP33-41 *in vitro*. An intracellular cytokine staining was performed and CD45.1+ CD8+ specific T cells (P14) of either P14 BM→WT or P14 BM→*DEE* mice were characterized, IFN-γ secreting cells are given as percentage of lymphocytes (n=2-4; pooled from two independent experiments).

3.3 Introduction of LCMV-GP minor antigen in hematopoietic stem cells leads to deletion of host specific CD8⁺ T cells after BMT

During minor antigen mismatched BMT, host specific CD8⁺ T cells were present despite of minor antigen expression in the host. The hypothesis was that only antigen expressed in the hematopoietic derived cells is able to induce deletion of host specific CD8⁺ T cells in this setting. To test this hypothesis, we mixed P14 bone marrow cells with *DEE* bone marrow cells in a 1:1 ratio and transferred them into *DEE* and WT mice. In both groups, host specific CD8⁺ T cells were deleted **(Figure 3.6)**

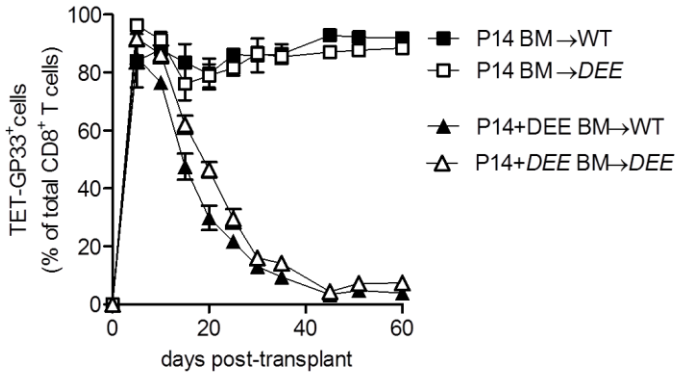

Figure 3.6 P14 T cell kinetic in blood of chimeric mice after mixed bone marrow transplantation. Percentage of GP33 specific CD8⁺ T cells (P14 T cells) was determined in blood by flow cytometry at indicted time points after BMT (n=5).

3.4 Recipients ignore minor antigen (LCMV-GP) expressed in cardiac graft

Heterotopic HTX was performed as murine SOT model. Donor heart was harvested from *DEE* mice and transplanted in WT mice **(Figure 3.7).**

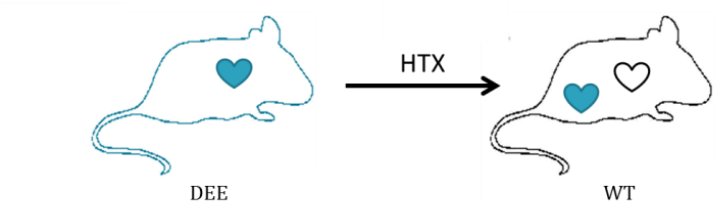

Figure 3.7 Model of heterotopic heart transplantation. GP33 expressing heart was harvested from *DEE* mice and transplanted in WT mice. As control, heart was harvested from WT mice and transplanted in WT mice.

Analyzing host versus graft reaction in SOT-setting showed that GP33 expression in the heart was completely ignored. Presence of minor antigen mismatch in syngeneic cardiac graft did not induce rejection in a time period of 100 days whereas allogenic hearts harvested from Sv/129 mice were rejected in WT mice within 10 days **(Figure 3.8)**. Sv/129 mice differ genetically from WT mice by MHC (H2 complex) class I and II alleles and the genetic background and were used as allograft rejection model (positive control).

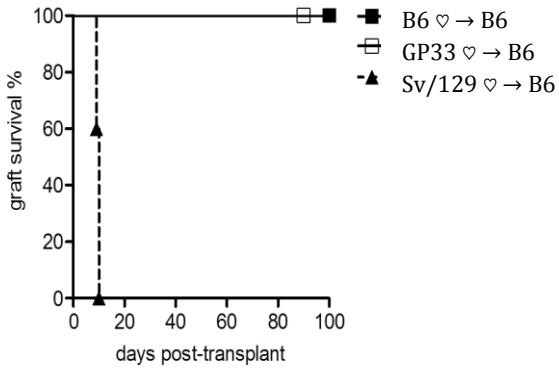

Figure 3.8 Graft survival curve after HTX. Graft survival was defined as persistence of heart beating. Graft survival was independent of survival of recipient mice. B6 (black square), *DEE* (GP33; white square) and Sv/129 (black triangle) hearts were harvested as donor hearts and transplanted in B6 (WT) mice. Heart beat was determined weekly by abdominal palpation (n=4-5).

Moreover, no development of GP33 specific CD8⁺ T cells could be detected in WT mice recipients transplanted with a syngeneic WT heart or a *DEE* heart carrying the LCMV-GP33 peptide **(Figure 3.9)**. No priming of CD8⁺ T cells took place. In conclusion, minor antigen mismatch was completely ignored by host derived WT CD8⁺ T cells, in this setting.

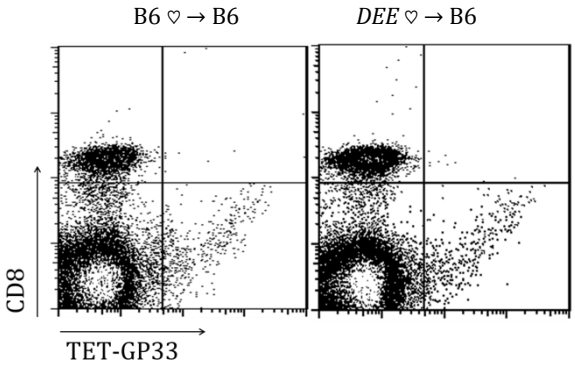

Figure 3.9 Tetramer staining of blood after HTX. At day 100 post-transplant blood from transplanted mice receiving either B6 (WT) heart or *DEE* (GP33) heart were analyzed for GP33 specific CD8⁺ T cells by flow cytometry. Dotplot was gated on lymphocytes. Left panel shows representative dotplot of B6 (WT) mice receiving B6 (WT) heart (n=3). Right panel shows representative dotplot of B6 (WT) mice receiving *DEE* heart (n=4).

3.5 Immunological tolerance in an LCMV model

WT mice received either naïve B6 heart or LCMV loaded heart. Effect of viral transmitted infection by HTX versus systemic (*i.v.*) LCMV infection post-transplantation has been analyzed as followed **(Figure 3.10).**

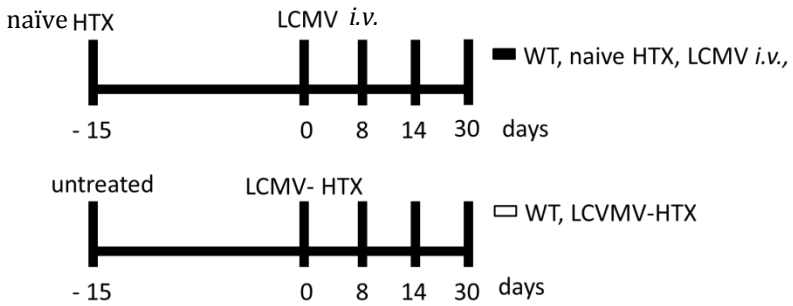

Figure 3.10 Experiment design. WT mice recipients received either a naïve heart transplant or LCMV loaded heart. WT mice receiving a naïve heart transplant were infected with LCMV strain WE intravenously.

3.5.1 LCMV carrier mice

To create LCMV carrier mice, *Tcrb*$^{-/-}$ mice which cannot mount an antiviral immune response because of TCR deficiency required for MHC class I expression, have been infected with 2 x 10^6 PFU of LCMV strain WE. Mice were then chronically infected and virus persisted in mice. Virus titer is shown and is comparable to LCMV-WE high dose infection **(Figure 3.11)**. LCMV carrier heart was used as donor heart which was severely infected with replicating virus. In the previous experiments **(Figure 3.5)** GP33 expression alone was not able to induce immune activation. The hypothesis was that only replicating virus was able to break immunological ignorance.

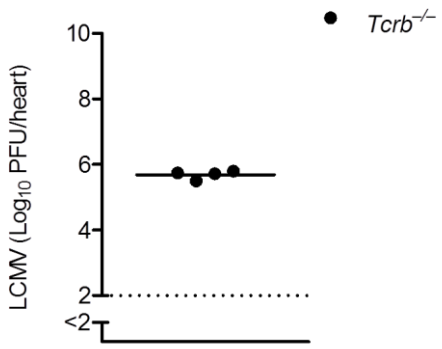

Figure 3.11 Virus titers in LCMV donor hearts. *Tcrb*$^{-/-}$ mice were infected with 2 × 10^6 PFU of LCMV strain WE. Graph shows viral titers in heart after 30 days post-infection (n = 4). Horizontal dotted lines designate the detection limit.

3.5.2 LCMV transmitted by HTX leads to T cell exhaustion in WT mice

In this setting, viral loaded hearts were transplanted in naïve mice and naïve hearts were transplanted in virus infected mice. The syngeneic grafts whether naïve or infected were not rejected. The hearts kept on beating and recipients survived. WT mice receiving chronically infected hearts became carrier mice; meaning, virus persisted in mice and spread from the transplanted heart to other tissue. Mice receiving naïve-HTX and were infected systemically with LCMV cleared the virus. Plaque assay of organs and heart transplant showed viral

persistence 30 days post-transplant **(Figure 3.12)** in LCMV-HTX mice but not in LCMV infected naïve-HTX mice.

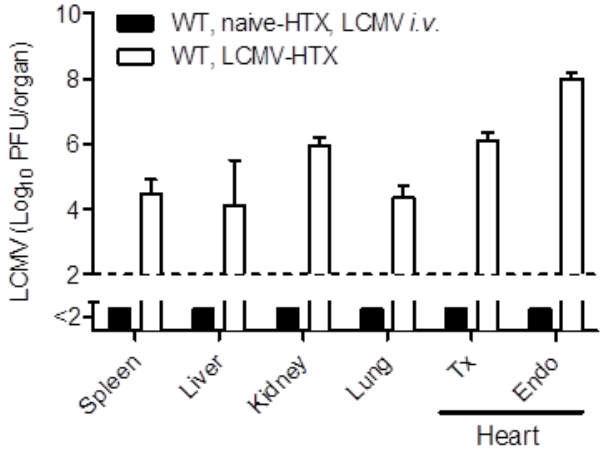

Figure 3.12 Virus titers in organs after LCMV-HTX. Organs have been removed 30 days post-transplant for Plaque Assay. White columns show virus titers in spleen, liver, kidney, lung, transplanted heart (Tx) and endogenous heart (Endo) removed from WT mice recipients receiving LCMV-loaded hearts (n=4). Control group (black columns) received naïve hearts and were infected with LCMV strain WE intravenously 15 days post-transplant. 30 days post-infection organs have been harvested for Plaque Assay (n=4). Horizontal dotted lines designate the detection limit.

Virus infection could not be controlled in LCMV-HTX mice. Analyzing virus specific $CD8^+$ T cells, which normally develop after LCMV-WE infection, revealed a reduced GP33 specific $CD8^+$ T cell number in blood **(Figure 3.13A)**. Furthermore viral specific response in form of effector cytokine production such as IFN-γ was reduced in blood **(Figure 3.13B)**. Together these data demonstrate anti-viral T cell exhaustion in chronically infected mice. T cell exhaustion is defined as a loss of effector function (Wherry, 2011; Yi et al., 2010). Gradual loss can be distinguished as stages which are determined by diminished or vanished production of effector cytokines such as IFN-γ, increased expression of inhibitory receptors and deletion of antigen-specific T cells. In addition, T cell exhaustion correlates with viral persistence.

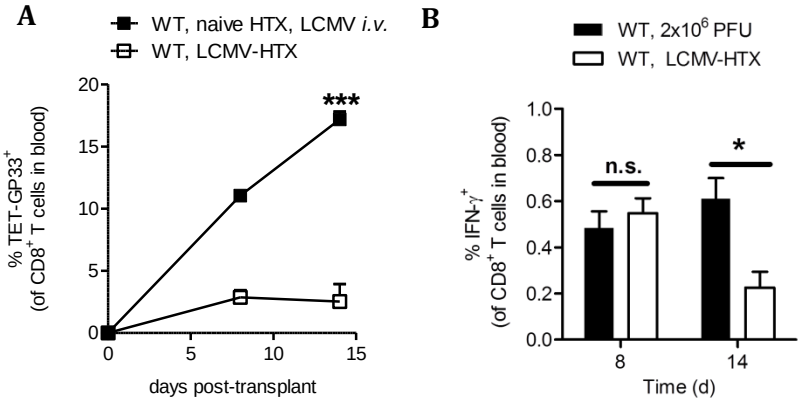

Figure 3.13 CD8⁺ T cell kinetic and IFN-γ production in blood. A) Percentage of GP33 specific CD8⁺ T cells was determined in blood by flow cytometry at indicted time points post-transplant or post-infection respectively. WT mice received LCMV carrier hearts (white squares; n=4). Control group consisted of WT mice which received naïve hearts and were infected *i.v.* with LCMV strain WE low dose 15 days post-transplant (black squares; n=4). Time is shown in days post-transplant and post-infection respectively. B) IFN-γ production was assessed by intracellular cytokine staining (ICS) in blood gated on CD8⁺ T cells. White columns represent WT mice receiving LCMV loaded hearts (n=4) at days post-transplant and as control, naïve WT mice infected with LCMV strain WE high dose at days 8 and 14 post-infection.

3.6 Breaking immunological tolerance

In the study reported here, minor antigen expression in hosts (*DEE* mice) receiving BMT, induced ignorance in donor BM derived minor antigen specific CD8⁺ T cells (P14 cells). Antigen expression in syngeneic graft (*DEE* heart) during SOT was ignored by the lymphoid system of the host (WT mice). Replicating antigen in transplant (LCMV- HTX) induced tolerance in naïve transplant recipients due to T cell exhaustion. Now the question occurred how to break this tolerance.

3.6.1 High frequencies of host specific CD8⁺ T cells induce limited GvHD after stimulation with LPS

Donor derived LCMV-GP minor antigen specific CD8⁺ T cells (P14) grafted in *DEE* mice remained ignorant, which means, these mice did not develop GvHD. However, innate immune activation via Toll-like Receptor (TLR) can have an impact on adaptive auto-reactive immune responses (K. S. Lang et al., 2006; K. S. Lang, Recher, Junt, et al., 2005).

Therefore, chimera mice which received P14 bone marrow were treated with lipopolysaccharides (LPS). LPS was injected intraperitoneally in P14 BM→WT mice and P14 BM→*DEE* mice 200 days after BMT. Serologically, LDH was measured as general marker for cell damage and transaminases as specific markers for liver disease. When LPS was administrated LDH was enhanced in P14 BM→*DEE* mice but not in P14 BM→WT mice, indicating cell damage took place **(Figure 3.14)**. Specific liver enzymes were in normal range. This suggests that LPS impacts on immune activation of host specific CD8⁺ T cells. However, these data show that even at high precursor frequencies of minor antigen specific CD8⁺ T cells acute GvHD is not induced.

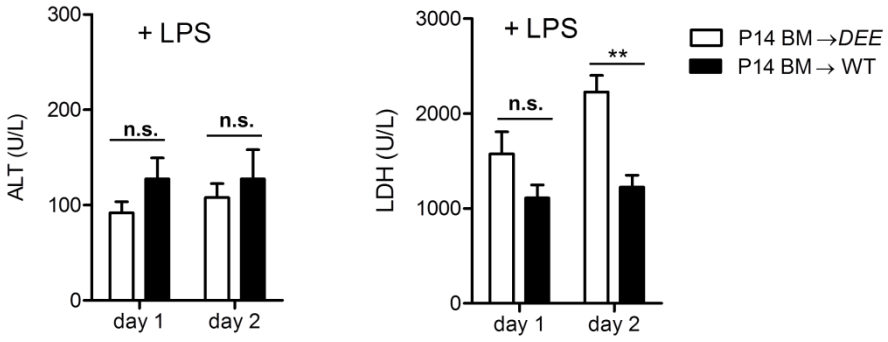

Figure 3.14 ALT and LDH measurement in serum after LPS challenge. P14 bone marrow was transferred in *DEE* and WT mice (white columns P14 BM in *DEE* mice; black columns P14 BM in WT mice (n=4-5) pooled from two experiments). 50µg LPS was injected intraperitoneally 200 days after BMT. 1 and 2 days after LPS challenge, serum was analyzed for ALT and LDH amount.

3.6.2 IL-10 deficient mice die after transplantation of LCMV infected hearts

Generation of GP33 specific CD8+ T cells in the presence of *DEE* transplanted heart did not take place due to T cell exhaustion **(Figure 3.8)**. Possible mechanisms of CD8+ T cell exhaustion during heart transplantation can vary. In this study, CD8+ T cell exhaustion led to tolerance of heart transplant and consequently to syngeneic graft survival. Interleukin-10 is known to play a crucial role for CD8+ T cell exhaustion during chronic infection (Blackburn et al., 2007; Brooks et al., 2006). Therefore, IL-10 deficient mice were used as recipient **(Figure 3.15)**.

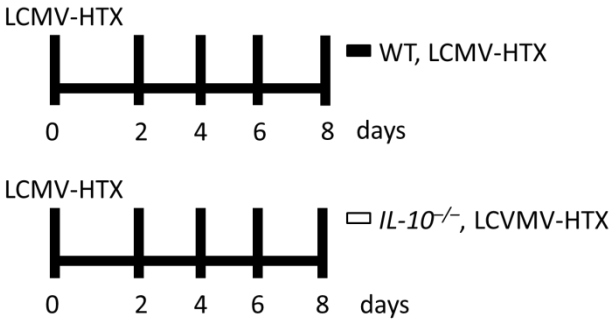

Figure 3.15 Experiment design. LCMV carrier hearts were transplanted in *IL-10*$^{-/-}$ mice recipients or in WT mice recipients. Time line shows the days post-transplant. At indicated time points, blood analysis was done by flow cytometry.

Interleukin-10 is an immunosuppressive cytokine which is secreted by various types of cells such as CD8+ and CD4+ T cells, B cells and APCs (Moore et al., 2001). When mice lack IL-10 during LCMV-HTX, they died **(Figure 3.16A)**. This can be due to high numbers of activated CD8+ T cells. Thus, infection with LCMV-WE intravenously in IL-10 deficient mice led to fast generation of LCMV specific CD8+ T cells (data not shown). When LCMV carrier hearts were transplanted into IL-10 deficient mice, higher numbers of CD8+ T cells could be detected in blood **(Figure 3.16B)**. Indeed, IL-10 was elevated in WT mice receiving an infected heart transplant **(Figure 3.17A).** Virus titer in blood was not affected by IL-10 loss **(Figure 3.17B)**.

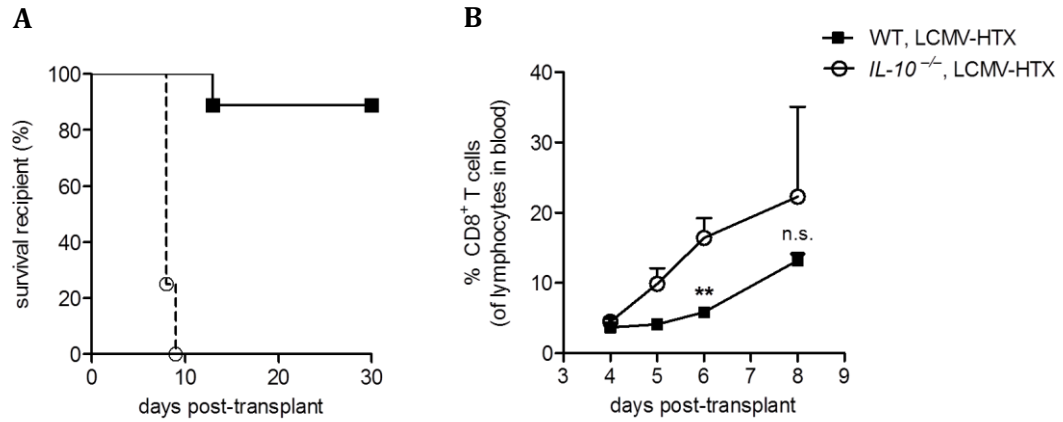

Figure 3.16 Survival curve and CD8⁺ T cell kinetic after LCMV-HTX. LCMV carrier hearts were transplanted in *IL-10*$^{-/-}$ mice recipients (white circle) and in WT mice recipients (black square). A) Mice were observed in a time period of 30 days. Survival of mice recipients after LCMV-HTX is shown in percentage (n=7-15; pooled from 2 to 3 experiments). B) CD8⁺ T cells were measured in blood by flow cytometry at indicated time points post-transplant (n=2-4).

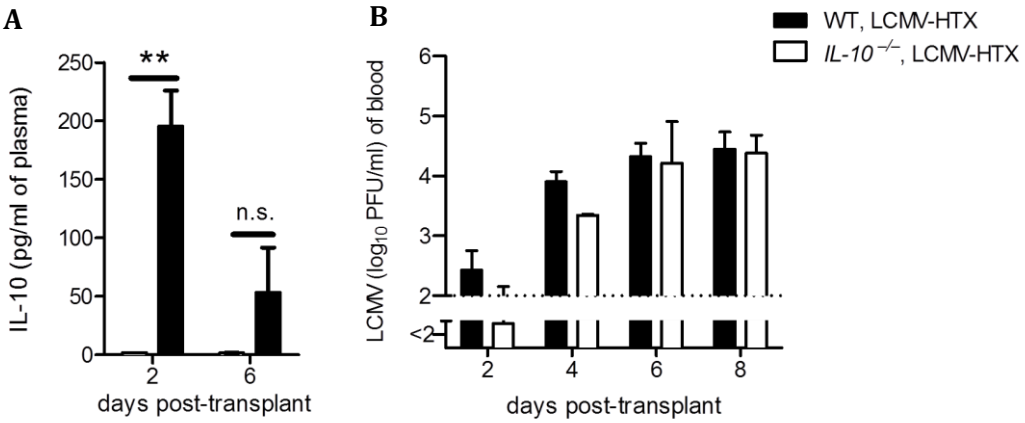

Figure 3.17 IL-10 and virus titers in plasma and blood after LCMV-HTX. LCMV carrier hearts were transplanted in *IL-10*$^{-/-}$ mice recipients and in WT mice recipients (n=2-4). A) IL-10 was measured in plasma by ELISA at day 2 and 6 post-transplant. B) Plaque Assay was performed from blood. Virus load was determined by plaque-forming units in blood at days post-transplant. Horizontal dotted lines designate the detection limit.

IL-10 deficient mice rejected the hearts within 10 days and heart enzymes were elevated in *IL-10*$^{-/-}$ mice **(Figure 3.18A)**. In addition, *IL-10*$^{-/-}$ mice became ill and revealed high levels of LDH and ALT indicating massive cell death in tissue and liver damage respectively **(Figure 3.18B)**. The mice revealed a systemic illness and died within 10 days due to immunopathology. Together these data show that

IL-10 was essential to prevent syngeneic graft rejection after transplantation of viral infected hearts.

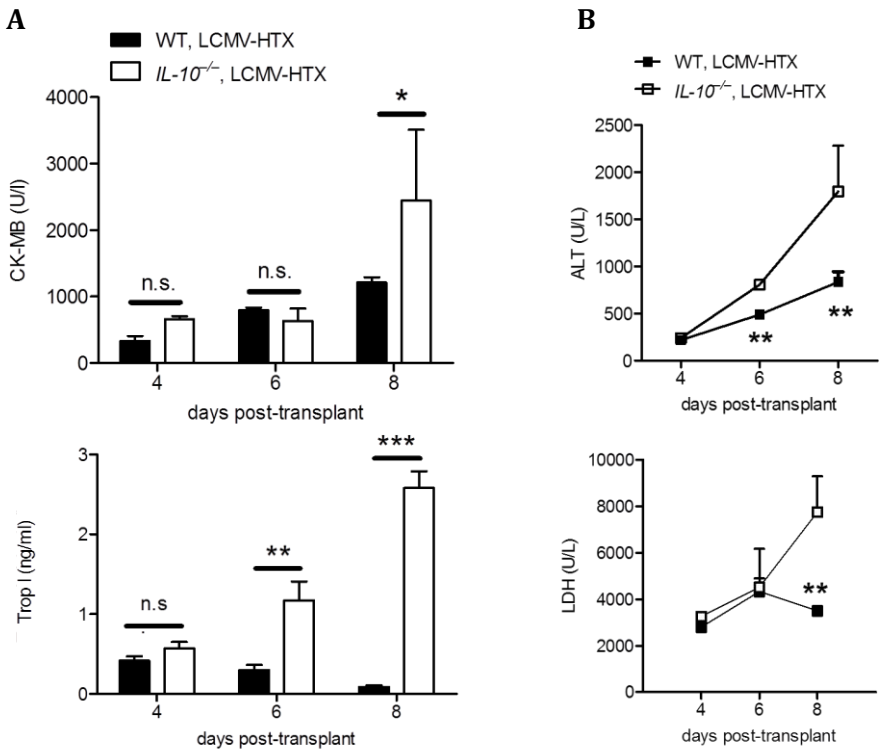

Figure 3.18 Analysis of heart and liver enzymes in sera after LCMV-HTX. LCMV carrier hearts were transplanted in *IL-10*$^{-/-}$ mice recipients and in WT mice recipients. A) CK-MB and Trop I (specific heart enzymes) were measured in sera at day 4, 6 and 8 post-transplant (n=2-4). B) ALT and LDH (marker for liver damage and dell death) were determined in sera at day 4, 6 and 8 post-transplant (n=2-4).

3.7 Graft rejection by memory T cells

Are memory T cells able to prevent virus persistence? Are they dependent on IL-10? Therefore, memory CD8^{+} T cells were generated in C57BL/6 mice by infecting mice with LCMV-WE intravenously. After priming with LCMV, mice generated a specific immune response. In a next step, LCMV carrier hearts were transplanted into LCMV infected mice **(Figure 3.19)**.

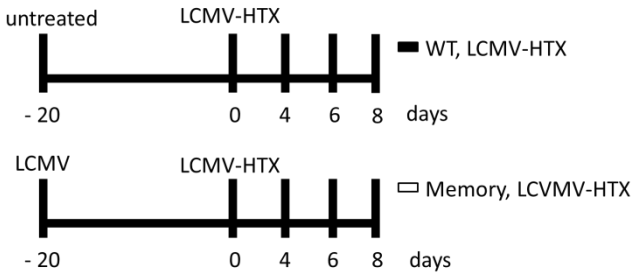

Figure 3.19 Experiment design. Naïve C57BL/6 (WT) mice were infected at least 20 days before HTX with 200 PFU of LCMV-WE intravenously and generated memory $CD8^+$ T cells. Memory mice or naïve C57BL/6 (WT) mice received LCMV infected hearts and blood analysis were made at indicated time points post-transplant.

3.7.1 Memory mice recipients reject LCMV infected heart transplant

Transplantation of LCMV infected hearts led to massive $CD8^+$ T cell expansion in memory mice **(Figure 3.20A)**. Memory $CD8^+$ T cells got highly activated as shown by down regulation of IL-7 receptor (CD127) and CD62L **(Figure 3.20B)**. Virus specific memory $CD8^+$ T cells could control LCMV **(Figure 3.21A)** and transplanted hearts were early rejected in memory mice **(Figure 3.21B)**. In line with the acute heart rejection, heart specific parameters, CK-MB and Trop I, raised early after transplantation **(Figure 3.21C)**. Histology revealed high numbers of infiltrating $CD8^+$ T cells in the heart (data not shown). In conclusion, we found that presence of memory $CD8^+$ T cells resulted in fast rejection of viral infected hearts, but virus could be controlled and the mice survived.

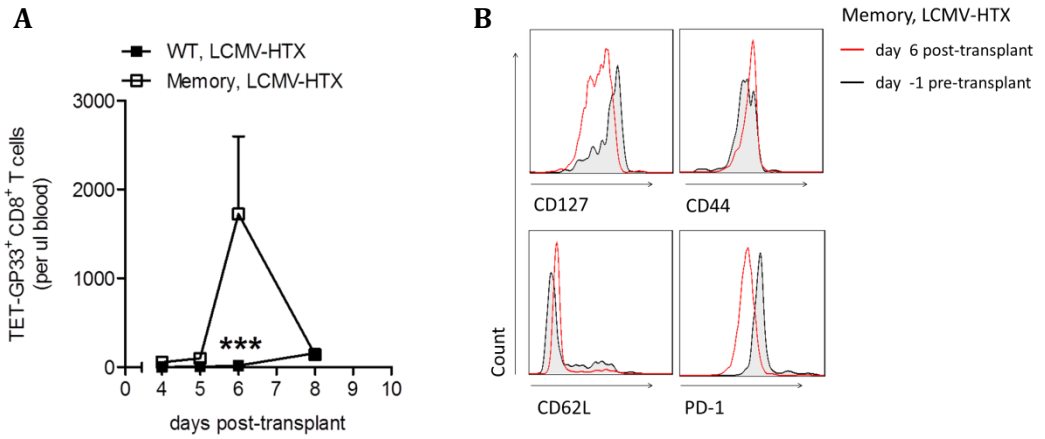

Figure 3.20 Characterization of Memory T cells. A) Graph showing frequency of T cells that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-GP33+) and for CD8 (CD8+) in blood of WT or LCMV infected recipients receiving LCMV loaded hearts shown in days post-transplant (n=3-5). B) Analysis of activation marker such as CD127, CD62L, CD44 and PD-1 are made by gating TET-GP33+ CD8+ T cells and shown in a histogram blot at day 6 post-transplant in comparison to day -1 pre-transplant in blood. One histogram blot is shown as a representative of each group.

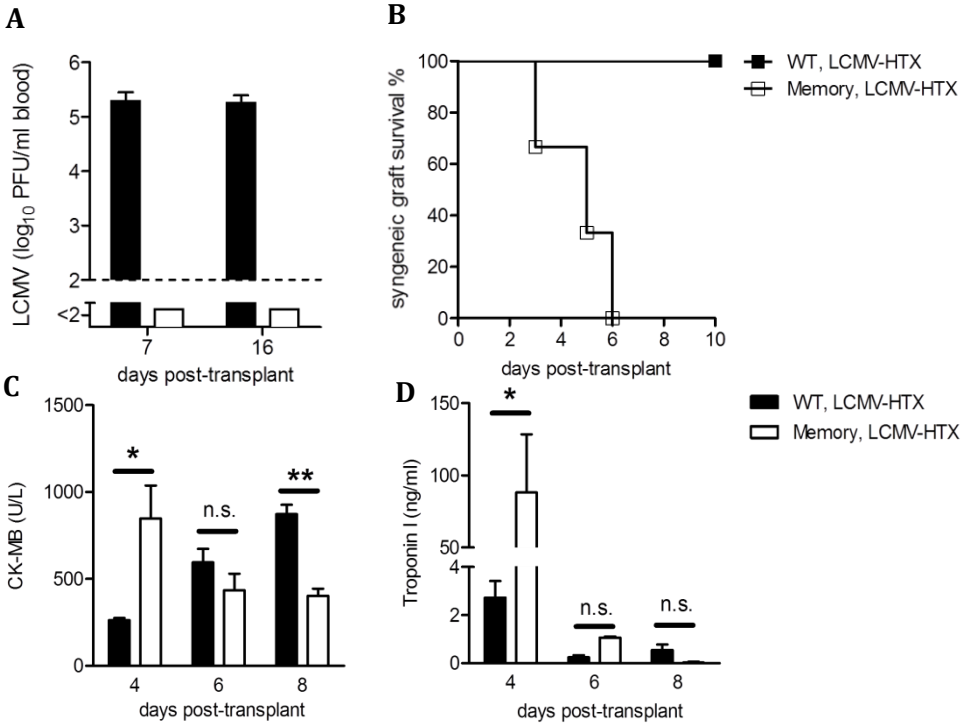

Figure 3.21 Virus control and transplant rejection in memory mice. LCMV carrier hearts were transplanted in memory mice recipients and in WT mice. A) Virus titers in blood were determined by plaque-forming units (PFU) in Plaque assay at days 7 and 16 post-transplant. Horizontal dotted lines designate the detection limit (n=4). B) Allograft survival curve after LCMV-HTX was determined by rest of heart beat (n=4-7). Allograft survival was independent of recipient survival. C) Measurement of heart enzymes (CK-MB and Trop I) were determined in sera (n=3-4).

4 Discussion

In the study reported here, the role of viral infection in transplantation medicine was assessed in a murine LCMV model. Two main experimental but clinical relevant conditions have been used: murine bone marrow transplantation and heterotopic heart transplantation. Minor antigens as well as viral infection influence outcome of patients undergoing BMT or SOT in fully matched donor and recipients. The LCMV-GP transgene was used as a peptide in a minor antigen mismatch transplantation model. In a next step, the role of replicating antigen was analyzed in heterotopic heart transplantation. LCMV transmitted by heart transplantation led to T cell exhaustion in WT mice and in consequence to syngeneic graft tolerance. Moreover, induction of host antigen (GP33) in hematopoietic stem cells led to deletion of host specific $CD8^+$ T cells after BMT.

4.1 Host specific $CD8^+$ T cells proliferate in presence of minor antigen in host after BMT

In the murine minor antigen mismatched bone marrow model we transplanted P14 bone marrow into *DEE* mice to analyze the activation of these host specific $CD8^+$ T cells. The recipients developed high frequencies of GP33-specific T-lymphocytes independent of antigen expression in the thymus and peripheral organs. Nevertheless, they did not develop lethal disease in form of GvHD. To confirm central tolerance induction by recognition of GP33 minor antigen and by T cell deletion we crossed P14 mice with *DEE* mice. Almost no GP33-specific $CD8^+$ T cell has been detected in peripheral organs. *DEE* mice express ubiquitously GP33 peptide in organs and transferring CFSE labeled mature but naïve P14 T cells into *DEE* mice led to significant T cell proliferation, suggesting that the minor antigen is detectable by the transgenic TCR of the P14 T cells. As described in previous publications proliferation of anti-host reactive $CD8^+$ T cells could be induced by minor antigen presentation in the host (Mori et al., 2005). It

remains unclear, whether presentation of minor antigens in the host can lead to ignorance and finally to reduced immunopathology in patients after BMT.

4.2 Triggering the innate immune system can lead to activation of host specific CD8+ T cells

We demonstrated that triggering the innate immune system with LPS could lead to significant cell damage in *DEE* recipients whereas WT recipients remained unaffected by GP33-specific CD8+ T cells. This is in line with the clinical situation where bacterial or viral infection (e.g. CMV) often induces GvHD (Ljungman, 2008). Especially for the human cytomegalovirus (HCMV), preexisting data show an association of viral infection with increased risk of aGvHD (Boeckh et al., 2004; Nichols et al., 2002). Interestingly, in a clinical trial it was shown that early replicative HCMV infection reduces the risk of leukemic relapse in patients with acute myeloid lymphoma (AML) (Elmaagacli et al., 2011). Though the underlying mechanism is not completely understood yet; a virus-induced abrogation of immune ignorance comparable to our murine model and even induction of graft versus leukemia (GvL) is a possible explanation for reduced risk of leukemic relapse after early replicative HCMV infection. Thus, beyond genetic haplotype matches the immune status on bacterial and viral infections of bone marrow donors might be helpful for GvHD risk evaluation, which is already performed for herpes viruses and HCMV (Pietersma et al., 2011).

4.3 Outlook for possible therapeutic methods in BMT

These data are in contrast to current models, suggesting that low frequencies of anti-host reactive CD8+ T cells result in development of GvHD (Desmarets et al., 2009; Schroeder et al., 2011). We found that in absence of secondary stimulation even high numbers of host-reactive T cells are ignorant. GvHD is often associated with GvL. GvL can result in complete control of malignant

cells after minor HLA mismatch. Interestingly efficient GvL can occur even in the presence of mild GvHD (Klymenko et al., 2011). The reasons for that are not well explained. From our data we would suggest that the presentation of leukemic antigens in primary lymphoid organs is crucial for the recognition by anti-host reactive $CD8^+$ T cells and for early elimination. Therefore, a promising approach to treat GvHD would be the inhibition of T cell egress to peripheral organs. This would potentially limit aGvHD, but still allow GvL. Such a preparation is Fingolimod (FTY720) which is licensed for treatment of relapsing remitting multiple sclerosis. Fingolimod prevents the egress of lymphocytes from secondary lymphoid organs thereby preventing migration of immune cells to the central nervous system (Chun et al., 2010). Fingolimod is not clinically tested in BMT. Even though its application seems attractive, Fingolimod has first to be evaluated in animal models since it reduces the peripheral lymphocyte count and acts as an immunosuppressant. In conclusion we demonstrated that high frequencies of anti-host reactive $CD8^+$ T cells can remain ignorant after engraftment in recipient and do not induce GvHD.

4.4 Immunized recipients have an increased risk for cardiac graft rejection

We mimicked the clinical situation of donor-derived viral infection in SOT in form of heterotopic heart transplantation in a mouse model. This system allows us to focus on recipients' immune response against virus infection transmitted through organ transplantation.

Clinically, CMV is a major problem in SOT (Razonable et al., 2013). CMV is a herpesvirus which is widely spread in human beings. Seroprevalence of CMV varies between 30 and 97% in the population (Bate et al., 2010; Cannon et al., 2010). Consequently, more than 50% of solid organ transplant recipients suffer from CMV infection in the first 3 months post-transplant. Seropositivity of donor and recipient play a crucial role in morbidity and mortality for the transplant recipient. CMV-seropositive donors and recipients are designated as D^+ and R^+

respectively. According to seroprevalence, different risk groups are categorized. In the D^+ and R^- (D^+/ R^-) group the recipient has the highest risk for CMV disease. The lowest risk for CMV disease is in the case of seronegativity in donor and recipient (D^-/R^-) (Ramanan et al., 2013). A CMV prophylaxis treatment is preserved for transplant recipients who are at highest risk for CMV disease (D^+/ R^-) and (D^+/ R^+). A retrospective study compared morbidity and mortality in four CMV donor/recipient serostatus categories (Harvala et al., 2013). As result, the group D^+/ R^+ showed the highest risk for mortality post-transplant, even though CMV disease occurs more often in D^+/ R^- . In line with our data, recipients who have been infected once before transplantation (R^+) show a strong immune response against the allograft (D^-) leading to rejection. In our study, LCMV infection via transplant (D^+) in naïve recipient (R^-) mice led to chronic virus infection due to $CD8^+$ T cell exhaustion. The grafts survived and showed only partial dysfunction.

4.5 Role of LCMV infection in human beings

Another example for donor-derived infection in humans is LCMV. LCMV is an arenavirus, which is very rare in human beings. An infection is usually asymptomatic in healthy persons and can be caused after close contact to house mice or hamsters (Childs et al., 1992; Childs et al., 1991). Some cases are known where LCMV was transmitted during transplantation. Donor-derived infection in several recipients led to death within three weeks after transplantation. The same viral strain could be detected in a cluster of patients, where seven of eight recipients died. The recipients revealed unspecific symptoms such as diarrhea, fever and systemic illness (Fischer et al., 2006). Normally, LCMV infection in non-immunosuppressed people is asymptomatic.

Immunosuppression after transplantation aims to reduce immune response by the adaptive immune system. Nevertheless, it encloses many side effects such as a higher risk of opportunistic infections, a heighten incidence of cancer and toxicity (Halloran, 2004). In our study, viral infected hearts were transplanted in

immunocompetent mice and were fully HLA-matched to focus specifically on $CD8^+$ immune response.

4.6 T cell exhaustion- a possible mechanism inducing tolerance?

A new perspective for tolerance in SOT is to understand $CD8^+$ T cell exhaustion. Little is known in the field of transplantation medicine. We know that T cell exhaustion limits the immune response against chronic infections and tumors. In case of recrudescence of exhausted T cells, clearance of infections and tumor regression can be induced (Blank et al., 2007).

Previous clinical studies effort to reactivate exhausted T cells as treatment for chronic infections or tumor disease (Sharpe et al., 2015). However, the mechanisms leading to T-cell exhaustion are unknown. Further, the role of T-cell exhaustion in transplantation or autoimmunity is not defined. Recently, *Sarraj et al* (Sarraj et al., 2014) showed that genetic deletion of selectin ligand for leucocyte migration leads to impaired T cell function. They focused on $CD4^+$ T cells and induced T cell exhaustion in a murine transplantation model by impaired migration capability. They determined T cell exhaustion by impaired effector cytokine production (IFN-γ), defective proliferation of $CD4^+$ T cells and higher PD-1 expression. Knock-out mice recipients providing impaired migration capability revealed T cell exhaustion and showed prolonged graft survival. T cells were not able to migrate in the MHC II mismatched heart transplant which in consequence led to permanent activation of T cells and then to T cell exhaustion. Graft survival was significantly increased. The data demonstrate that T cell exhaustion led to tolerance of allograft.

The fact that IL-10 is an immunoregulatory cytokine that is associated with T cell exhaustion, is in line with the above finding (Blackburn et al., 2007). Its function during virus infection is described to be immunosuppressive by limiting cytokine production and proliferation of $CD8^+$ and $CD4^+$ T cells leading to viral persistence. A variety of cells can produce IL-10 such as T cells, B cells and APCs (Blackburn et al., 2007; Moore et al., 2001). Clinical studies demonstrated that

polymorphisms linked with increased IL-10 production are associated with increased susceptibility to chronic HCV infection and increased severity of chronic HBV infection (Knapp et al., 2003; Paladino et al., 2006; Persico et al., 2006). Vice versa, polymorphisms with reduced expression of IL-10 correlate with a slower progression of AIDS in HIV- infected patients (Shin et al., 2000). Its role in transplantation medicine is poorly understood. As IL-10 is known for its anti-inflammatory response and immunosuppressive role, it should abet graft survival. Indeed, IL-10 inhibits ischemia/reperfusion injury (Deng et al., 2001), extends allograft survival and function (Cypel et al., 2009; Feng et al., 1999; Zuo et al., 2001) and is essential for the action of regulatory T cells mediating tolerance at least in some transplant models (Hara et al., 2001). In our model, IL-10 deficient mice receiving LCMV loaded heart died early after transplantation. Loss of IL-10 in recipients showed a systemic immune response with immunopathology indicating that absence of IL-10 prevents T cell exhaustion. Its immunoregulatory function in virus infection through organ transplantation helps tolerating the allograft.

This changed in case of memorized mice. Memory $CD8^+$ T cells were capable to control donor-derived LCMV infection but induced an acute graft rejection. Memory T cells play a major role in acute and chronic allograft rejection. Pre-transplant frequency of donor-specific alloreactive memory T cells in recipient correlates with the risk of long-term allograft rejection (Heeger et al., 1999). Previous experimental models demonstrate their potential alloreactivity. They even showed that cross-reactivity can lead to alloreaction defined as heterologous immunity (van den Heuvel et al., 2015). In our case replication competent LCMV in heart transplant was rejected in memory mice. These facts emphasize the significance of donor-derived viral infection. Immunized patients exposed to viral load facing possible alloreaction have to be treated virus specifically (e.g. CMV). It decreases significantly graft survival in SOT. IL-10 therapy in case of unexpected donor-derived viral infection could be a potential immunotherapy in SOT.

5 Summary

Graft versus host disease (GvHD) occurs in 40% of cases with patients having a MHC I matched bone marrow transplantation (BMT). Mechanisms causing this disease remain to be studied. Here we used a $CD8^+$ T cell transgenic mouse strain (P14/$CD45.1^+$) and *DEE* mice bearing the foreign antigen (LCMV-GP33-41) to study mechanisms of tolerance in donor derived host specific $CD8^+$ T cells after BMT. We found that host reactive $CD8^+$ T cells were not negatively selected in the thymus and developed comparably to host non-specific $CD8^+$ T cells. Host specific $CD8^+$ T cells ignored the antigen expressed ubiquitously by host cells but they could be activated *ex vivo* via LCMV-infection. Lipopolysaccharides (LPS) induced transient cell damage in *DEE* mice bearing host specific $CD8^+$ T cells, suggesting that induction of host inflammatory response could break this ignorance. In conclusion, we found that after BMT host specific $CD8^+$ T cells ignore antigen in recipients and that they are only deleted when host antigen is present in the hematopoietic system. Moreover, LPS-induced immune activation contributes to induction of alloreactivity of host specific $CD8^+$ T cells after BMT. Unexpected transmissions of viral pathogens during solid organ transplantation (SOT) can result in severe, life-threatening diseases in transplant recipients. Immune activation contributes to disease onset; however mechanisms balancing the immune response against transmitted virus infection through organ transplantation remain unknown. Here, we found, using LCMV, that transplantation of LCMV infected hearts led to exhaustion of virus specific $CD8^+$ T cells, viral persistence in organs and survival of graft and recipient. Genetic depletion of IL-10 resulted in a strong immune activation, graft dysfunction and death of mice, suggesting that IL-10 was a major regulator of $CD8^+$ T cell exhaustion during SOT. In the presence of memory $CD8^+$ T cells, virus could be controlled; however sufficient antiviral immune response resulted in rejection of transplanted heart. In conclusion, we found that virus transmitted by SOT cannot be controlled by naive recipients due to IL-10 mediated $CD8^+$ T cell exhaustion which thereby prevented immunopathology and graft failure whereas memory mice recipients were able to control the virus and induced graft failure.

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7 Abbreviation

Ab	antibody
ALT	alanine transferase
AML	acute myeloid lymphoma
APC	antigen presenting cell
BMT	bone marrow transplantation
CAV	cardiac allograft vasculopathy
CD	cluster of differentiation
CFSE	carboxy-flourescein-succinimidyl-ester
CK-MB	creatinine kinase of type muscle brain
CMV	cytomegalovirus
DMEM	dulbecco`s modified eagle medium
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
GvHD	graft versus host disease
GvL	graft versus leukemia
GP	glycoprotein
Gy	gray
HBV	hepatitis B virus
HCV	hepatitis C virus
HLA	human leukocyte antigen

HTX	heart transplantation
i.p.	intraperitoneal
i.v.	intravenous
IVC	inferior vena cava
IL	interleukin
IMDM	iscoves modified dulbeccos medium
IFN-γ	interferon gamma
LCMV	lymphocytic choriomeningitis virus
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MC57	mouse fibrosarcoma cell line
NAT	nucleic acid testing
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PeCy7	phycoerythrin-cyanin-7
PerCP	peridin-chlorophyll
PFU	plaque forming units
PSG	penicilin-streptomycin-glutamine
qRT-PCR	quantitative real time PCR
RIC	reduced intensity conditioning
SOT	solid organ transplantation
TCR	t cell receptor
TLR	toll-like receptor

Trop I	troponine I
WT	wild type

8 Tables and Figures

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10 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.